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Human p68 kinase exhibits growth suppression in yeast and homology to the translational regulator *GCN2*

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The human p68 kinase is an interferon-regulated enzyme that inhibits protein synthesis when activated by double-stranded RNA. We show here that when expressed in *Saccharomyces cerevisiae*, the p68 kinase produced a growth suppressing phenotype resulting from an inhibition of polypeptide chain initiation consistent with functional protein kinase activity. This slow growth phenotype was reverted in yeast by two different mechanisms: expression of the p68 kinase N-terminus, shown to bind double-stranded RNA *in vitro* and expression of a mutant form of the α-subunit of yeast initiation factor 2, altered at a single phosphorylatable site. These results provide the first direct *in vivo* evidence that the p68 kinase interacts with the α-subunit of eukaryotic initiation factor 2. Sequence similarity with a yeast translational regulator, *GCN2*, further suggests that this enzyme may be a functional homolog in higher eukaryotes, where its normal function is to regulate protein synthesis through initiation factor 2 phosphorylation.

Key words: dsRNA/eIF-2α phosphorylation/*GCN2*/growth inhibition/p68 kinase

Introduction

The translational machinery involved in the regulation of protein synthesis is highly conserved from yeast to humans (Merrick *et al.*, 1990; Prat *et al.*, 1990). There appears to be extensive similarity of amino acid sequence (usually >99%) in most characterized initiation factors within the mammalian species, while sequence identity compared with yeast is >35% (Merrick *et al.*, 1990). Rates of protein synthesis are tightly regulated and respond rapidly to metabolic changes within the cell. Altered translation rates occur at fertilization, during mitosis, upon nutrient starvation or stress and following treatment with hormones or growth factors (Hershey, 1989). Protein phosphorylation is known to control the rate of protein synthesis most frequently during initiation at the level of 43S complex formation [binding of ternary complex (initiator met-tRNA–eIF-2–GTP) to 40S ribosomal subunit] or at the level of 48S complex formation (binding of mRNA to the 43S complex) (Proud, 1986; Sonenberg, 1990). Protein kinases that phosphorylate the α-subunit of eIF-2 (eIF-2α) have long been known to inhibit protein synthesis (Safer, 1983; Proud, 1986). The phosphorylation of eIF-2α inhibits the recycling of this factor when it is bound to GDP, resulting in the loss of ternary complex formation, thus inhibiting protein synthesis initiation (Safer, 1983; Pain, 1986; Proud, 1986). In both yeast and humans, a decrease in the rate of formation of 43S preinitiation complexes occurs during amino acid starvation (Vaugham *et al.*, 1971; Pain *et al.*, 1980; Tzamarias *et al.*, 1989). This suggests that eIF-2α kinases may play a common role in the control of protein synthesis during nutritional stress.

In mammalian cells, a protein kinase of M_r 68 000 (p68 kinase) has been shown specifically to phosphorylate eIF-2α *in vitro* when activated by double-stranded RNA (dsRNA) (Hovanessian, 1989). The p68 kinase contains all of the 11 conserved kinase subdomains that can be discerned through the alignment of 65 different members of the protein kinase family (Meurs *et al.*, 1990). In addition to the consensus sequence in subdomain VI that indicates serine/threonine specificity, this protein kinase contains a 32 amino acid insert upstream of this subdomain which we have suggested is involved in specific substrate recognition (Meurs *et al.*, 1990). Although the precise cellular function of the p68 kinase is not known, it is present in most cell types at basal levels and increases following viral infection or treatment of cells with interferon (Galabru and Hovanessian, 1987; Meurs *et al.*, 1990).

The α-subunit of eIF-2 in the yeast, *Saccharomyces cerevisiae*, shares 80% amino acid homology with its human equivalent when conservative substitutions are considered (Cigan *et al.*, 1989). Based on this conservation, we reasoned that the human p68 kinase might function in yeast and thus provide a system amenable to genetic analysis. Here we report that the expression and activation of the human p68 kinase in *S.cerevisiae* produces a growth inhibition that can be reverted by co-expression of a regulatory N-terminus of the p68 kinase or a mutant α-subunit of eIF-2. Previously, a yeast protein kinase, GCN2, was observed to exhibit a similar slow growth phenotype when overexpressed on a multiple-copy plasmid (Tzamarias and Thireos, 1988). We note sequence similarity of the p68 kinase to this yeast regulator of protein synthesis, and propose that the p68 kinase represents the mammalian equivalent of GCN2 and is involved in the control of higher eukaryote protein synthesis in response to environmental stimuli.

Results

The human p68 kinase expressed in S.cerevisiae inhibits growth

We constructed p68 kinase plasmids in the yeast vector pEMBLyex4 in order to express the human dsRNA dependent protein kinase in yeast. Inducible expression in this vector is controlled by the *GAL10–CYCI* hybrid promoter (Figure 1). The wild-type expression plasmid (called yex86), when introduced into yeast cells and subsequently grown on galactose as the sole carbon source, produced a slow growth phenotype. This was observed either as very small colonies on solid medium (Figure 2a) or a much increased (4.6-fold) doubling time in liquid media (Figure 2b). The slow growth phenotype suggested to us that the p68 kinase was expressed and possibly phosphorylating a critical yeast protein required for growth. To test this hypothesis, a site-directed mutant of the p68 kinase, 6M, was generated by substituting an arginine residue for a lysine residue at amino acid position 296 in subdomain II of the catalytic domain of the p68 kinase. This invariant lysine is present in all protein kinases that have been characterized to date and appears to be involved directly in the phosphotransfer reaction, possibly mediating proton transfer (Kamps and Sefton, 1986; Hanks *et al.*, 1988). Accordingly, all substitutions of this amino acid have shown a loss of protein kinase activity (Hanks *et al.*, 1988). *In vitro* phosphorylation of the 6M mutant protein, produced using a rabbit reticulocyte lysate system, showed much reduced kinase activity (data not shown). The 6M mutant gene was placed in the vector pEMBLyex4, forming yex6M (Figure 1) and expressed in yeast. Normal growth on galactose was observed (Figure 2, yex6M), indicating that the p68 kinase-induced slow growth phenotype resulted specifically from the expression of protein kinase activity.

The presence of biologically active p68 kinase in yex86 containing cells was confirmed by immunoprecipitation of the expressed p68 kinase using a monoclonal antibody (mAb 71/10) to the human p68 kinase, followed by *in vitro* phosphorylation of the p68 kinase-bound proteins (Figure 3C and D). Immunoblotting using this antiserum was also performed to determine whether p68 kinase protein was produced (Figure 3B). As shown in Figure 3C, kinase

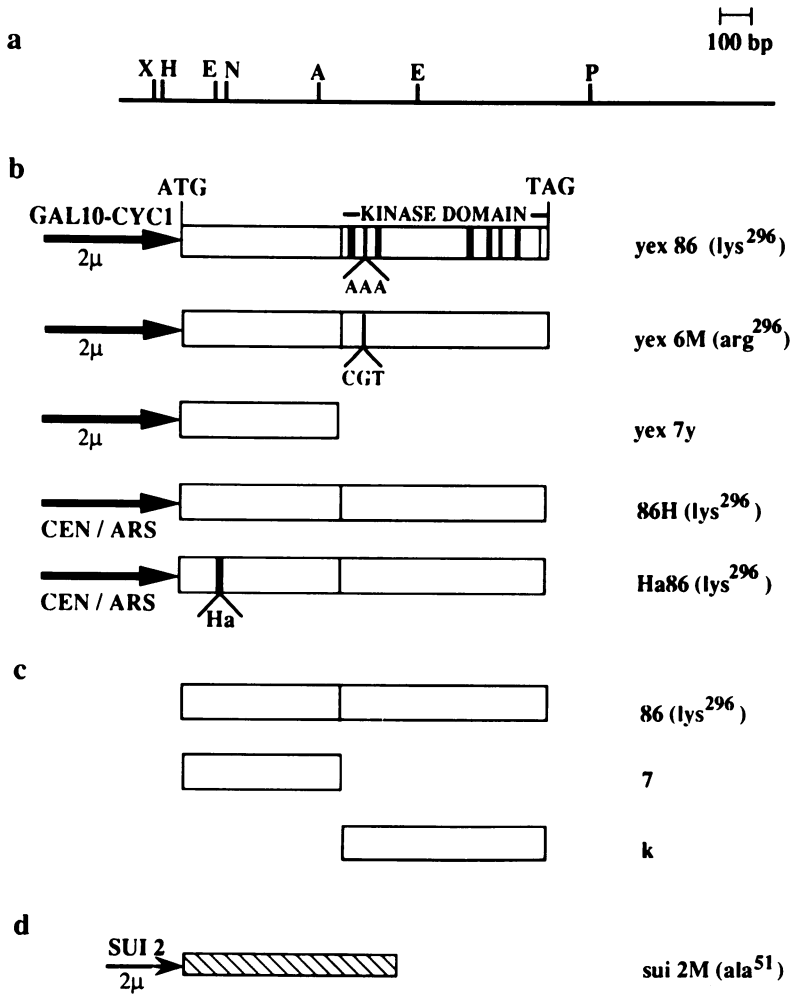

Fig. 1. Schematic diagram of expression plasmids used in this study. (**a**) Restriction map of the p68 kinase cDNA showing the location of restriction sites used for plasmid constructions. Letter designations for restriction sites are: X, *Xba*I; H, *Hind*III; E, *Eco*RI; N, *Nco*I; A, *Acc*I; P, *Pst*I. (**b**) Wild-type (yex86 lys296) and mutant (yex6M arg296) p68 kinase proteins expressed under the control of the yeast *GAL10/CYCI* promoter on a 2μ or a centromeric (CEN/ARS) vector. The translational start and termination sites, highly conserved kinase subdomains, sequence change of lys296 (AAA) to arg296 (CGT) in subdomain II and insertion site of the hemagglutinin (Ha) epitope are indicated. (**c**) *In vitro* translated wild-type (86 lys296) and truncated (7, k) p68 kinase proteins. (**d**) Mutant yeast eIF-2α (sui2M ala51) protein expressed constitutively on a 2μ vector under the control of its endogenous promoter.

activity was observed in the absence of exogenous dsRNA only with the wild-type p68 kinase (yex86). Thus, the growth inhibition observed with wild-type p68 kinase is correlated with autophosphorylated p68 kinase, whereas little kinase activity is associated with the arg296 mutant yex6M (Figure 3C), which grew normally (Figure 2, yex6M). No p68 kinase protein could be detected by immunoblotting of extracts prepared from the wild-type expressing yeast strain (Figure 3B, yex86) although protein from the mutant yex6M was observed (Figure 3B, yex6M). This significant reduction in the level of wild-type protein kinase, although clearly detectable by phosphate labeling, suggested that the activity of the p68 kinase might be directly affecting yeast protein synthesis. To verify this, polysome profiles from yeast extracts containing pEMBLyex4, the mutant 6M and the wild-type p68 kinase were compared (Figure 4). Panels A and D, representing the vector and mutant kinase respectively, showed nearly identical polysome profiles in the presence of both glucose and galactose. The presence of increasing large polysomes was evident as well as a small 80S peak; this likely represented a non-specific association between the 40S and 60S ribosomal subunits. In contrast, the wild-type p68 kinase (panel B) exhibited a decreasing number of larger polysomes with a significant accumulation of 80S particles in the presence of galactose. Similar polysome profiles have been observed with *sui1* and *sui2* temperature sensitive mutants grown at a non-permissive temperature (Yoon and Donahue, 1992) and both of the respective gene products have been directly implicated in translation initiation. The simplest explanation for this aberrant polysome profile observed with the yex86 strain is that the galactose induced expression of the wild-type p68 kinase leads to the inhibition of translation initiation. Consistent with this, p68 kinase activity from yeast extracts was able to recognize and phosphorylate human eIF-2α *in vitro* (Figure 3D, yex86). Thus, the expression of p68 kinase under the control of a strong inducible promoter resulted in the production of activated kinase, which was growth suppressive in yeast due to an inhibition of translation initiation.

An endogenous activator of the p68 kinase is present in yeast

The p68 kinase expressed in yeast is activated as revealed by its ability to autophosphorylate and phosphorylate the substrate eIF-2α, *in vitro* (Figure 3C and D, yex86). The p68 kinase is cAMP and cGMP independent and requires the presence of Mn^{2+}, ATP and low concentrations of dsRNA for activation and autophosphorylation (Galabru and Hovanessian, 1987). As a result, in mammalian cells, the p68 kinase is often activated by viral infection (Hovanessian *et al.*, 1987; Taylor and Grossberg, 1990). However, in addition to dsRNA, other polyanionic molecules such as heparin, poly L-glutamine, dextran sulfate and chondroitan sulfate are also able to activate the protein *in vitro* (Galabru and Hovanessian, 1987), although their significance *in vivo* remains to be determined. The ability of the p68 kinase to function in yeast implies that an endogenous activator must be present inside the cell.

Most laboratory strains of *S.cerevisiae* contain dsRNA viruses (ScVs), which are the most likely candidates for activators of the p68 kinase in our experiments. These are typical fungal dsRNA viruses in that each is encapsidated in a virus-like particle (Wickner, 1986; Schmitt and Tipper, 1990) which is similar to the outer capsid shell that surrounds the dsRNA genome of the human reovirus, a member of the family, Reoviridae (Giatini *et al.*, 1984). Both reovirus and yeast ScV RNAs have been shown to activate the p68 kinase *in vitro* while reovirus infection has also been shown to activate the p68 kinase *in vivo* (Torrence *et al.*, 1981; Nilsen *et al.*, 1982; M.Katze, unpublished observation). Alternatively, dsRNA generated from the asymmetric transcription of the yeast plasmid DNA might behave as an activator. Transient transfection of plasmid DNA in mammalian cells has previously shown a localized activation of the p68 kinase (Akusjarvi *et al.*, 1987). In order to determine the presence of any endogenous activator, we prepared yeast extracts and used them to activate immunoprecipitated p68 kinase from mammalian cells. The results indicate that the mammalian p68 kinase can be activated with yeast extracts (Figure 5, lanes 4 and 7). The amount of p68 kinase activation using pEMBLyex4 transformed yeast extracts was approximately twice that

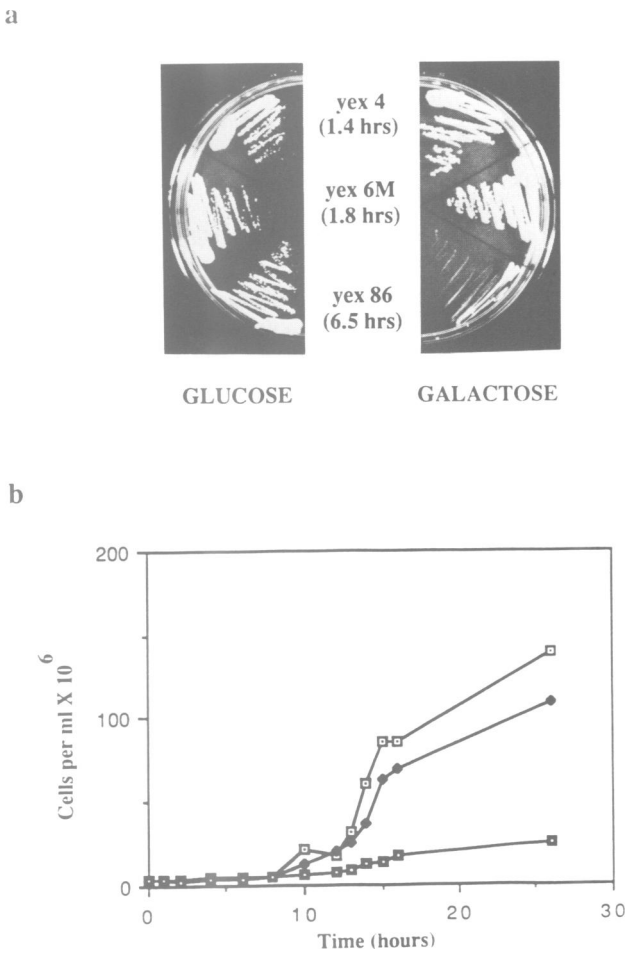

Fig. 2. Induction of wild-type p68 kinase causes slow growth in *S.cerevisiae*. (**a**) Growth of transformed yeast strains containing yex4, site-directed (lys296−arg296) p68 kinase mutant, yex6M and wild-type p68 kinase yex86. Cells were grown for 2 days at 30°C on agar containing synthetic medium lacking uracil with 2% glucose or galactose as the sole carbon source. Doubling times for transformants are indicated in parentheses. (**b**) Growth curves of transformed yeast strains; yex4 (—⊙—), yex6M (—◆—) and yex86 (—■—) grown at 30°C in synthetic medium lacking uracil and containing 2% galactose.

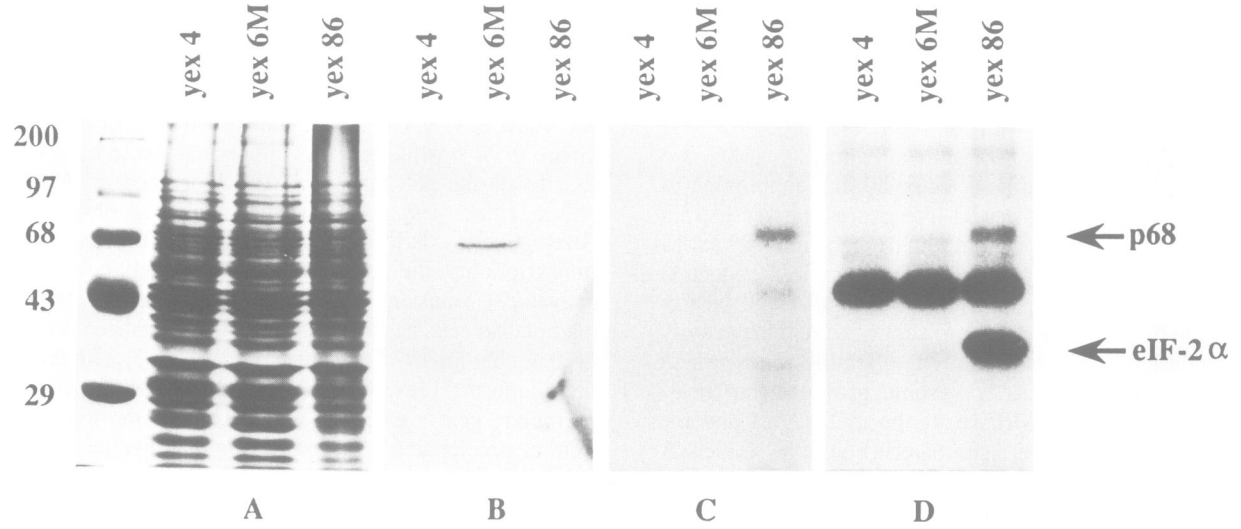

Fig. 3. Expression and phosphorylation of the p68 kinase in *S.cerevisiae*. Total cellular proteins were extracted from yeast transformants after 12 h of growth in galactose synthetic medium lacking uracil and analyzed by SDS−PAGE. (**A**) Expression of yeast proteins monitored by Coomassie brilliant blue staining of the gel. (**B**) Immunoblotting of expressed kinase proteins using p68 kinase monoclonal antibody (71/10). (**C**) *In vitro* autophosphorylation assay of expressed kinase proteins in the presence of [γ-^{32}P]ATP. (**D**) *In vitro* phosphorylation assay of substrate eIF-2α. Arrows indicate the position of the p68 kinase and eIF-2α. Positions of molecular weight markers (kDa) are indicated at the left side of the figure.

observed with the parental yeast extracts (as indicated by densitometric scanning, data not shown). This suggested that either an endogenous yeast activator or plasmid dsRNA could contribute to p68 kinase activation in our experiments.

The N-terminal region of the p68 kinase binds dsRNA in vitro

To investigate this activation further, we utilized a domain of the p68 kinase which was able to bind dsRNA *in vitro*. We initially localized this domain using poly I:poly C, a synthetic dsRNA analog, previously shown to bind and activate specifically the p68 kinase in mammalian cells (Hovanessian and Riviere, 1980; Galabru *et al.*, 1989; Meurs *et al.*, 1990). An analysis of two truncated mutants of the p68 kinase revealed striking differences in their ability to bind to poly I:poly C cellulose (Figure 6). The wild-type p68 kinase, 86, and the truncated mutant 7 (amino acids 1−256) were both able to bind poly I:poly C cellulose. However, no binding to the mutant k (amino acids 264−550) containing only the catalytic domain was observed, implying that a region involved in regulating kinase activity by binding to dsRNA is located in the N-terminus of the p68 kinase. This result has also been recently confirmed using viral activators (Katze *et al.*, 1991). Similarly, binding of the p68 kinase monoclonal antibody (mAb 71/10) was restricted to the N-terminus suggesting the presence of an antigenic epitope in this region of the protein (Figure 6). Thus, the p68 kinase structurally resembles other protein kinases (Hunter, 1987) in its organization of separate regulatory and catalytic domains.

The identification of a dsRNA binding domain suggested to us that this domain might be able to antagonize activation of the wild-type p68 kinase in yeast by binding dsRNA (endogenous activator) *in vivo*. Accordingly, the truncated mutant of the p68 kinase, yex7y was co-expressed with the wild-type p68 kinase yeast transformant, 86H (Figure 1). The results (Figure 7a and b) indicate that overexpression of the N-terminal portion of the p68 kinase (yex7y) reversed the slow growth phenotype that was imparted by the wild-type p68 kinase; this is shown by improved growth on galactose agar medium and reduced doubling time in liquid medium (Figure 7a and b, 86H/yex7y). Since growth had been restored to the strain 86H/yex7y, we expected to detect by immunoblotting a 68 kDa protein. Although the smaller N-terminal protein was present, the larger product of 86H/yex7y could not be detected (Figure 7c, panel B). This observation suggested that the amount of p68 kinase being produced was low and possibly below the detection limit of the Western blotting assay used here. To increase the specificity for detection of only the wild-type p68 kinase in an *in vitro* phosphorylation assay, the wild-type kinase, 86H, was 'tagged' with a peptide sequence corresponding to the hemagglutinin (Ha) of the influenza virus (Field *et al.*, 1988). Insertion of this peptide epitope at the N-terminus was found to produce no detectable change in the function of the wild-type kinase in yeast (data not shown). This fusion protein could now be specifically immunoprecipitated with monoclonal antibody raised against the Ha peptide, therefore preventing any cross reactivity between the truncated protein and the wild-type kinase. *In vitro* kinase assays (Figure 7c, panel C, left side) revealed a substantial decrease in autophosphorylation activity between the yeast strains expressing only the wild-type p68 kinase (Ha86/yex4) and the truncated N-terminus (Ha86/yex7y). These data demonstrate that *in vivo* expression of a regulatory N-terminal protein in yeast was able to reduce p68 kinase phosphorylation, resulting in a reversal of the slow growth phenotype.

Reversion of the slow growth phenotype in yeast by a mutant eIF-2α

Eukaryotic initiation factor 2 is composed of three non-identical subunits, α, β and γ which function early during translational initiation by forming a ternary complex of GTP and met-tRNA that subsequently binds to the 40S ribosomal subunit and mRNA (Safer, 1983; Pain, 1986; Proud, 1986). The phosphorylation of a serine (ser) residue located near the N-terminus of the mammalian α-subunit has been

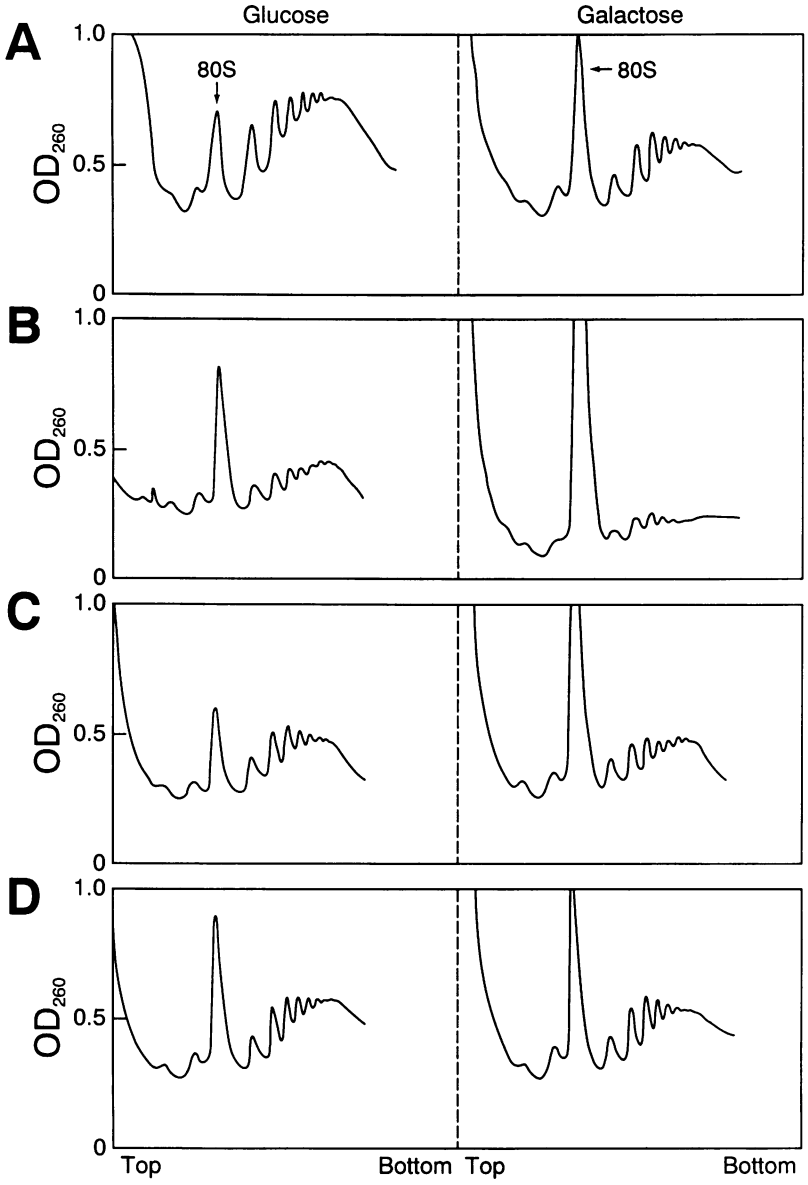

Fig. 4. Polysome analysis of p68 kinase expressing yeast strains. Yeast strains were grown in 2% galactose for 6 h and extracts were fractionated by sucrose gradient centrifugation. Gradients are plotted as OD_{260} units versus fraction (1.2 ml) number from the top to the bottom of the gradient. The left side of each panel represents growth in glucose while the right side of each panel represents growth in galactose. Polysome profiles of transformed yeast strains; yex4 (panel **A**), yex86 (panel **B**), 86H/sui2M (panel **C**), yex6M (panel **D**).

correlated with an inhibition of protein synthesis (Colthurst *et al.*, 1987). A systematic mutagenesis of six serine residues within the first 60 amino acids of the human eIF-2α subunit indicated that ser51 was the only site of phosphorylation that is involved in the repression of protein synthesis (Colthurst *et al.*, 1987; Pathak *et al.*, 1988). As mentioned above, the yeast and mammalian eIF-2α subunits are highly conserved; within the region proposed to be phosphorylated in mammalian cells, the amino acid sequence identity is 92% when conservative residues are considered (Cigan *et al.*, 1989). This high degree of similarity suggests that hyperphosphorylation of yeast eIF-2α by an as yet unidentified protein kinase could regulate protein synthesis initiation in yeast. As translational initiation was being affected in yeast strains expressing the p68 kinase, it seemed likely that the mechanism responsible for the growth inhibition was phosphorylation of yeast eIF-2α by the p68 kinase. In order to determine whether eIF-2α inactivation could be the cause of the observed slow growth phenotype, we analyzed the effect of a ser51 to ala51 mutant of yeast eIF-2α (sui2M) that was co-expressed in yeast with the p68 kinase (Figure 1). Expression of the sui2M mutant reversed the p68 kinase induced growth inhibition as observed by improved growth on galactose agar medium and a reduced doubling time (2.7-fold) in liquid medium (Figure 7a and b). In addition, polysome profiles of the cotransformed strain revealed a normal sedimentation pattern of 80S polysomes in comparison with the wild-type p68 kinase expressing yeast strain (Figure 4; compare panels C,A with B). Overall, these results demonstrate that the expression of sui2M can restore growth to strains previously growth inhibited by expression of the p68 kinase. Improved growth of these co-transformants allowed us to detect the expresssion of the p68 kinase by immunoblotting and *in vitro* phosphorylation

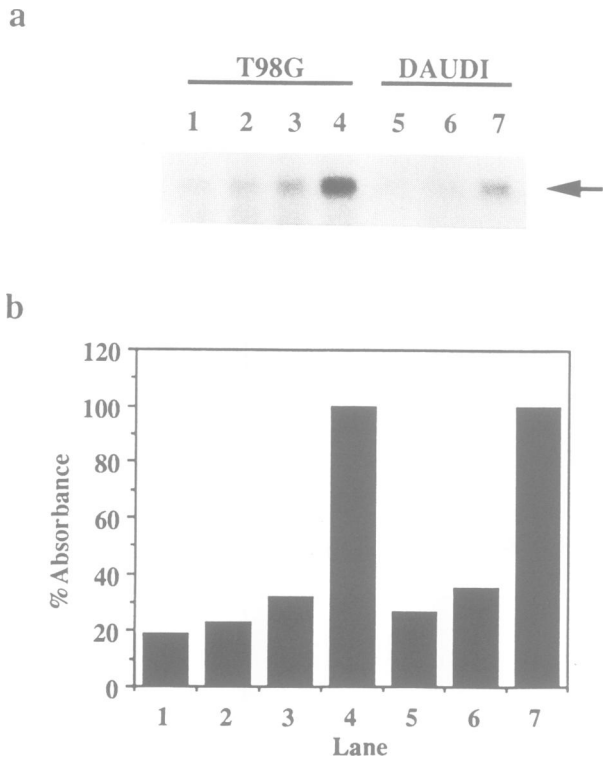

Fig. 5. *In vitro* activation of the mammalian p68 kinase using yeast cellular extracts. (**a**) Total cellular extract (200 μg) from T98G and Daudi cells was immunopreciptated with p68 kinase monoclonal antibody, activated with increasing amounts of pEMBLyex4 transformed yeast extract and subjected to an *in vitro* phosphorylation assay. Amount of yeast extract added was 0 μg (lanes 1 and 5), 4.9 μg (lane 2), 49 μg (lanes 3 and 6) and 245 μg (lanes 4 and 7). The arrow indicates the position of the p68 kinase. (**b**) Densitometric quantitation of the autophosphorylated band from (a).

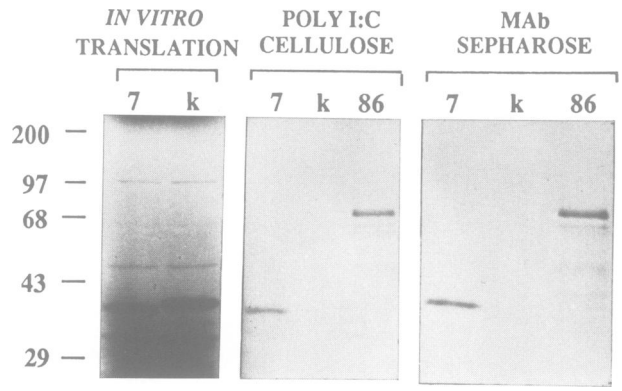

Fig. 6. *In vitro* expression and binding of truncated p68 kinase proteins to poly I:poly C cellulose and mAb–Sepharose. *In vitro* translation products containing the wild-type (86) and only the amino (7) or carboxyl (k) regions of the p68 kinase were labeled with [^{35}S]methionine and resolved by SDS–PAGE in the left panel. Translation products were incubated with poly I:C cellulose (middle panel) and p68 kinase monoclonal antibody (right panel), washed and subjected to SDS–PAGE and autoradiography. Positions of the molecular weight markers in kDa are indicated on the left side of the figure.

(Figure 7c, panels B and C, sui2M). As equal amounts of protein were loaded onto the gels used for Western blotting and autophosphorylation, the inability to detect wild-type p68 kinase in the 86H/yex7y revertant appears to have been a sensitivity problem if one compares the intensity of the 86H/sui2M autophosphorylated band with the amount of protein detected by Western blotting. Thus, we have shown that growth inhibition in yeast by the p68 kinase can be reverted by expression of an eIF-2α mutant altered at a single serine residue at position 51, which by analogy to human eIF-2α, can be phosphorylated.

Discussion

We have presented the first direct evidence that *in vivo* expression of the human p68 kinase can inhibit growth by blocking polypeptide chain initiation in yeast; this mechanism of inhibition correlates well with the known ability of the p68 kinase to phosphorylate ser51 in the smallest subunit (α) of eIF-2. The growth reversion exhibited by yeast strains expressing a mutant eIF-2α at position 51 confirms a specific interaction with the p68 kinase and suggests that this protein may be involved in the regulation of cellular growth at the level of protein synthesis initiation. Interferons have long been recognized as potent anti-proliferative agents having anti-tumor effects on a variety of human malignancies (Nanus *et al.*, 1990; Markovic and Murasko, 1991). The exact cellular mechanisms contributing to these effects have not yet been defined, however, the observation of growth suppression in yeast due to p68 kinase expression provides supporting evidence that directly implicates this interferon regulated gene in the growth inhibitory properties of interferon. Thus, interferons and translational regulators such as the p68 kinase may act as potential tumor suppressor genes. In this regard, it is interesting to note that the overexpression of eukaryotic protein synthesis initiation factor-4E (eIF-4E) has produced malignant transformation in several cell types (Lazaiis-Karatzas *et al.*, 1990).

Overexpression of the p68 kinase N-terminus also reversed the slow growth phenotype in yeast. However, based on the levels of p68 kinase protein produced in the reverted yeast transformants (see Figure 6c, panel B), this mechanism of reversion differed from that of the eIF-2α mutant and is probably not involved in the restoration of protein synthesis initiation. While this mechanism remains to be determined, it is possible that the N-terminal region is involved in sequestering activator or perhaps, directly interfering with the function of the catalytic domain. Since the N-terminus of the protein contains many hydrophilic and basic residues that may interact with dsRNA (Meurs *et al.*, 1990) or act as pseudosubstrate sequences (Soderling, 1990), auto-inhibition is possible. This is further supported by the conservation of charged residues in this region in both human and mouse kinase species (G.-S.Feng, K.L.Chong, A.Kumar and B.R.G.Williams, submitted). Experiments are now in progress to determine the significance of these homologies and their possible involvement in the N-terminus reversion of slow growth. The identification of amino acids constituting the dsRNA binding domain of the p68 kinase using co-expression in yeast should ultimately determine whether there exists a novel and specific motif for the binding of dsRNA.

Previously, overexpression of the p53 oncogene (Fields and Jang, 1990) and the yeast genes, *GCN2* or *GCN4* (Tzamarias and Thireos, 1988) produced slow growth in yeast. Both GCN2 and GCN4 are intimately involved in the translational control of amino acid biosynthesis in yeast, a regulatory system that is activated during amino acid starvation (Hinnebusch, 1988). The GCN2 protein kinase

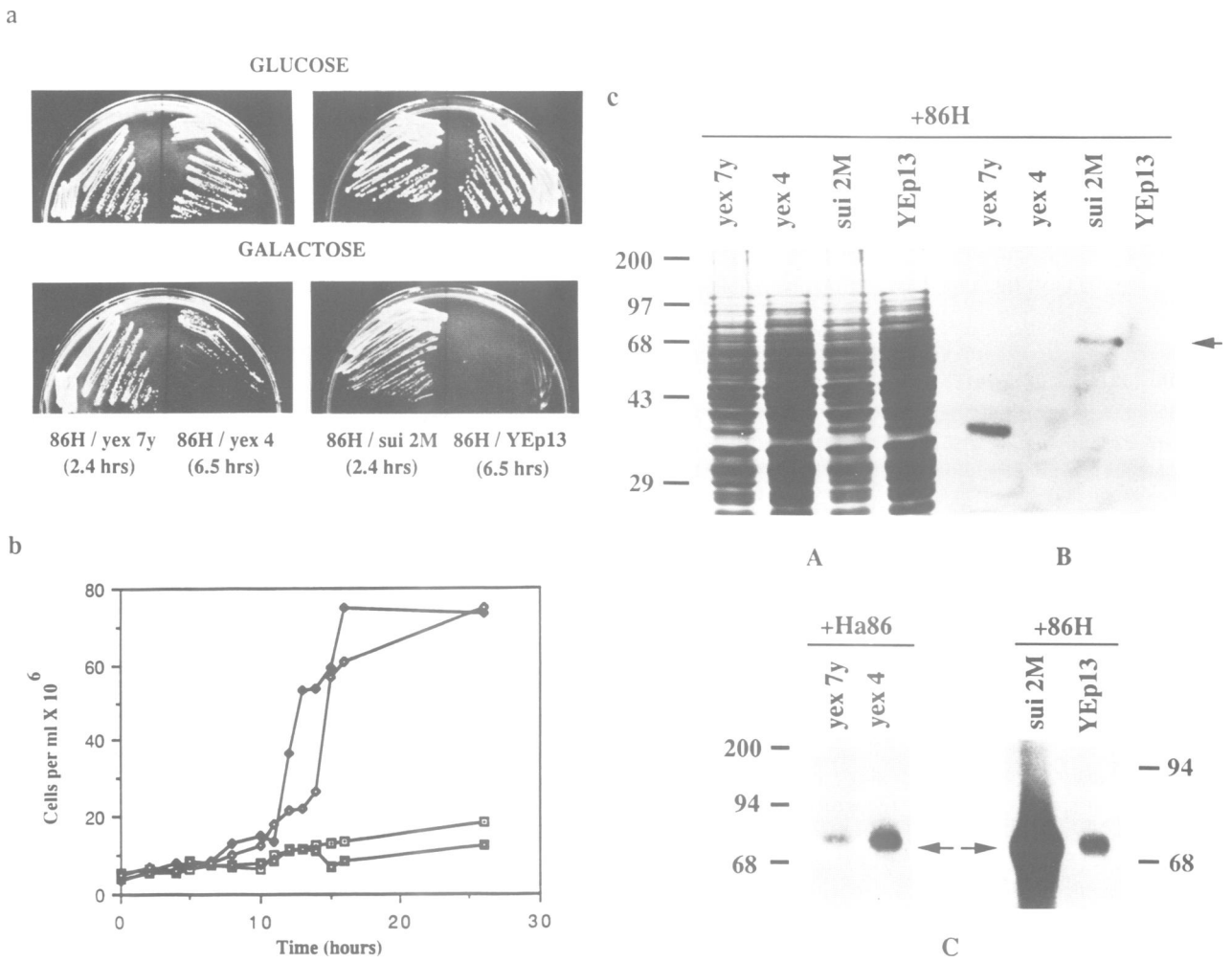

Fig. 7. Reversion of the slow growth phenotype in yeast by expression of the p68 kinase N-terminus (yex7y) or an eIF-2α mutant (sui2M). (**a**) Plate phenotype of co-transformed yeast strains containing 86H/yex7y, 86H/yex4 and 86H/sui2M, 86H/YEp13. Co-transformants were grown on agar containing synthetic medium lacking histidine and uracil or histidine and leucine with 2% glucose or galactose as the sole carbon source. Doubling times for co-transformants are indicated in parentheses. (**b**) Growth curves of co-transformed yeast strains, 86H/yex7y (—◆—) or 86H/yex4 (—⊡—) and 86H/sui2M (—□—) or 86H/YEp13 (—◆—), grown in synthetic medium lacking appropriate amino acids and induced with 2% galactose at 30°C. (**c**) Equal amounts of cellular extract prepared from yeast co-transformants (150 mg) were analyzed by SDS–PAGE. (**A**) Expression of p68 kinase proteins in yeast co-transformants was monitored by Coomassie brilliant blue staining of the gel. (**B**) Expressed p68 kinase proteins were detected by immunoblotting using p68 kinase monoclonal antibody. (**C**) Yeast extracts were immunoprecipitated with Ha monoclonal antibody (left side) or p68 kinase monoclonal antibody (right side) and subjected to an *in vitro* phosphorylation assay. Co-transformants contain either wild-type p68 kinase 86H or 86H containing Ha epitope (Ha86) as indicated. Arrows indicate the position of the p68 kinase. Positions of the molecular weight markers in kDa are as indicated.

is required for the expression of *GCN4* (Tzamarias and Thireos, 1988), a transcriptional activator of *GCN2* and other amino acid biosynthetic genes (Hinnebusch, 1988). GCN2 contains a kinase domain juxtaposed to a region with similarity to both prokaryotic and eukaryotic histidyl-tRNA synthetases (Wek *et al.*, 1989). These enzymes are able to bind uncharged tRNA that accumulates during starvation conditions (Clemens, 1990) to form amino-acyl tRNA. The histidyl-tRNA synthetase-related region of GCN2 may be involved in the activation of the kinase domain, since mutations in this region inactivate kinase function (Wek *et al.*, 1989). A substrate for GCN2 has not yet been identified, however indirect evidence suggests that eIF-2α may be involved (Tzamarias and Thireos, 1988; Clemens, 1990). Recently, mutations in the structural genes encoding the α or β subunits of eIF-2 were shown to stimulate induction of the GCN4 transcription factor by a mechanism that was independent of GCN2 (Williams *et al.*, 1989). Since the expression of *GCN2* is required for the translational derepression of *GCN4* (Tzamarias and Thireos, 1988), this observation implied that the function of the GCN2 protein kinase might be to disrupt eIF-2α or β, resulting in the synthesis of GCN4. Similarly, conditions that inhibited overall peptide chain initiation by modifications to eIF-2 led to enhanced levels of *GCN4* expression (Tzamarias and Thireos, 1988). Thus, eIF-2α may be the major target for phosphorylation by the GCN2 protein kinase. It would therefore be of interest to determine if the slow growth phenotype exhibited by GCN2 could be reversed by overexpression of an eIF-2α mutant.

A comparison of the amino acid sequence of GCN2 (Wek *et al.*, 1989) with the p68 kinase reveals a strong degree of sequence identity (38%) between the two catalytic domains (data not shown). In addition to the conservation of protein kinase subdomains, GCN2 and the p68 kinase share a striking nine amino acid region of homology (Figure 8) with two

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                IV                                                                          *
p68  316  DHU NIV QYHSCWEGVDYDPE.........................................TSDDSLESSDYDP.ENSKNSS  355
p65  291  NHV NIV HYNGCWDGFDYDPE...................................................H.SMSDTS.  333
GCN2 582  NHQ YVV RYYAAWLEEDSMDENVFESTDEESDLSESSSDFEENDLLDQSSIFKNRTNHDLDNSNWDFISGSGYPDIVFENSS  653
HRI  225  QHP NIV GYHTAWI.EHVHVHVQADRVPIQLPSLEVLSDQEEDRDQYGVKNDASSSSSIIFAEFSPEKEKSSDECAVEQNNK  304

p68  356  R...........................................SKTKC.................L F IQM EF C D  370
p65  334  R...........................................YKTRC.................L F IQM EF C D  332
GCN2 654  RDDENEDLDHD..TSSTSSSESQDDTDKESKSIQNVPRRRNFVKPMTAVKKKST...............L F IQM EY C E  723
HRI  305  LVNYTTNLVVRDTGDFDSSTDRQDNGSIVDRQLLFGHNSDVEEDFTSAEESSEEDLSALRHTEVQYHLM L H IQM QL C E  383

                   *                                       VII
p68  371  ..KGTLEQWIEKRRGEKLDKVLALELFEQITK.........GVDYIHSKKLIHRDLKPSNI FLVD  424
p65  333  ..KGTLEQWMRNRNQSKVDKALILDLYEQIVT.........GVEYIHSKGLIHRDLKPGNI FLVD  386
GCN2 724  NRTLYDLI.HSENLNQQR.DEYWRLFRQ.ILE.........ALSYIHSQGIIHRDLKPMNI FIDE  776
HRI  384  LSLW.DWIAERNRRSRECVDESACPYVMVSVATKIFQELVE GVFYIHNMGIVHRDLKPRNI FLHG  447
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Fig. 8. A sequence comparison of the kinase insert region of the p68 kinase with p65, GCN2 and HRI. Pairwise alignments made using the BestFit alignment program (Devereux *et al.*, 1984) were incorporated manually into this multiple sequence alignment. Catalytic subdomains are boxed with a thin black line while identical residues in the nine amino acid sequence homology are boxed with a thick black line. Gaps are indicated by dots; numbers on the side refer to amino acid residues. The asterisks mark the location of the p68 kinase insert.

recently identified protein kinases, the mouse homolog of the human p68 kinase, p65 (Feng *et al.*, submitted) and the heme regulated kinase, HRI (Chen *et al.*, 1991). This sequence homology is located in the kinase insert region of the p68 kinase previously suggested by analogy to other kinases to be involved in specific substrate recognition (Meurs *et al.*, 1990). While the significance of this sequence is not yet clear, we and others (Ramirez *et al.*, 1991) suggest the possibility that this sequence confers upon these kinases an ability to phosphorylate a common substrate, eIF-2α. Preliminary evidence suggests that only the boxed residues are important in p68 kinase–eIF-2α interactions (K.L.Chong and B.R.G.Williams, unpublished data). Certainly, the report of an 11 amino acid peptide within the kinase insert of the platelet-derived growth factor receptor that confers binding specificity to the phosphatidylinositol 3-kinase lends credence to this hypothesis (Escobedo *et al.*, 1991). From the analysis above, the similarities between the p68 kinase and the yeast GCN2 protein kinase are impressive. Structurally, both contain highly related catalytic domains and regulatory RNA binding domains that are probably involved in kinase activation. Functionally, both are inducible protein kinases that are thought to phosphorylate eIF-2α. Based on these common features, we believe that the p68 kinase might be a human functional homolog of GCN2 and possibly be regulated in a similar manner.

In Figure 9, we show a model that describes the activation of the p68 kinase and its role in the regulation of polypeptide chain initiation. In response to an environmental stress (viral infection, nutritional deprivation), the accumulation of an RNA molecule such as dsRNA or uncharged tRNA could induce an activator of the p68 kinase or activate the kinase directly. Once activated, phosphorylation of eIF-2α would inhibit protein synthesis, therefore reducing the consumption of amino acids and the amount of activator. The system would again become balanced by the activation of phosphatases involved in reversing the modification of specific phosphorylation events. The identification of phosphatases specific to eIF-2α and the p68 kinase (Pain, 1986; Szyszka *et al.*, 1989) and the ability of tRNA to

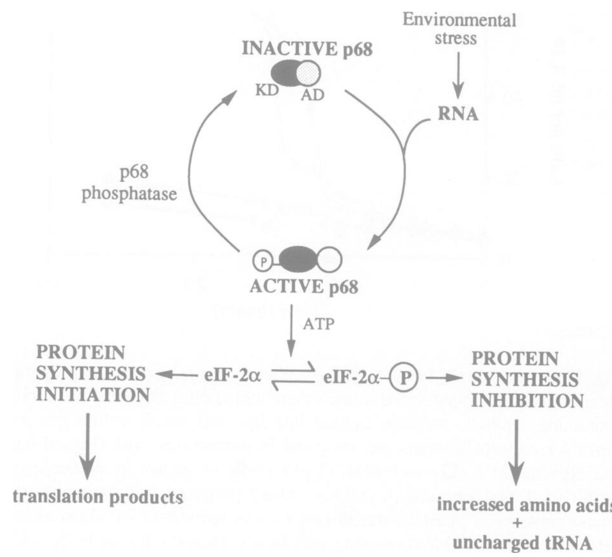

Fig. 9. A model for the regulation of polypeptide chain initiation by the human p68 kinase. In response to an environmental stress, an RNA molecule (dsRNA or ssRNA such as tRNA) could induce an activator of the p68 kinase or activate the kinase directly by binding to its N-terminus (AD—activation domain). Once activated, phosphorylation of eIF-2α by the p68 kinase catalytic domain (KD—kinase domain) would inhibit protein synthesis. Phosphatases involved in reversing the modification of specific phosphorylation events would then become activated.

activate purified mammalian p68 kinase *in vitro* (M.Katze and A.G.Hovanessian, unpublished observation) suggest that such a mechanism could exist *in vivo*.

A role for the p68 kinase in sensing increased levels of uncharged tRNAs could account for its low basal levels in most mammalian cells. Its remarkable similarity to GCN2 predicts that a similar pathway controlled by a GCN4-like transcriptional activator might exist in higher eukaryotes. It is conceivable that loss of p68 kinase function could occur at many points along the activation pathway; either through mutations in the kinase activator or in the protein molecule itself. The effect of such a loss would impair a vital control

of protein synthesis and possibly result in the dysregulation of other potential p68 kinase targets. The use of the *S.cerevisiae* expression system detailed here should provide a powerful tool for verification of this model through further structural analysis of the p68 kinase and identification of alternative cellular substrates.

Materials and methods

Plasmid constructions

The p68 kinase expression plasmids yex86 and yex6M were created by inserting a 1.8 kb *Xba*I–*Pst*I cDNA fragment (Meurs *et al.*, 1990) into the yeast expression vector, pEMBLyex4 (Cesareni and Murray, 1985), which carries the *URA3* gene. The site-directed mutant, yex6M (lys296–arg296), was created using a mutant oligonucleotide and conventional mutagenesis techniques (Kunkel *et al.*, 1987). The N-terminal of the p68 kinase, located on a 838 bp *Hin*dIII–*Acc*I cDNA fragment (Meurs *et al.*, 1990), was cloned into the yeast vector pEMBLyex4 and the transcription vector pGEM-3Z (Promega). The 963 bp C-terminal *Acc*I–*Pst*I cDNA fragment was similarly cloned into the vector pGEM-3Z. The centromeric expression vector for the wild-type p68 kinase was obtained by removing the galactose promoter and the cDNA insert from yex86 with *Apa*I–*Sna*BI. The resulting fragment was ligated to a *Sma*I digested pRS303 plasmid (Sikorski and Hieter, 1989) carrying a *HIS3* gene to yield plasmid 86H. The plasmid Ha86 was created in several steps, initially by inserting the oligonucoeotide pair

5′-CATG GGG TAC CCA TAC GAT GTT CCA GAT TAC GCT AGC ATG-3′

3′-CCC ATG GGT ATG CTA CAA GGT CTA ATG CGA TCG TAC-5′

into a unique *Nco*I site within the kinase plasmid pBS 86 (Meurs *et al.*, 1990). An *Xba*I–*Pst*I cDNA fragment was then cloned into pEMBLyex4 and an *Apa*I–*Sna*BI fragment containing the galactose promoter and the Ha-kinase insert was cloned into a *Sma*I digested pRS303 to produce Ha86. The mutant yeast eIF-2α expression plasmid (sui2M) was obtained by subcloning a 2.7 kb genomic *Bam*HI fragment (Cigan *et al.*, 1989) into a 2μ vector (YEp13) carrying a *LEU2* gene. Site directed mutagenesis of *sui2* (ser51–ala51) was performed using conventional techniques (Kunkel *et al.*, 1987).

Expression of the human p68 kinase in S.cerevisiae

Expression plasmids were introduced into the haploid yeast strain, W303-1a (*MAT***a**, *can*1-100, *his*3-11,15, *leu*2-3,112, *trp*1-1, *ura*3-1, *ade*2-1) using the lithium acetate procedure (Becker and Guarente, 1991). Transformed yeast strains were grown to stationary phase in synthetic medium (Sherman, 1991) containing 0.1% glucose and lacking only uracil, histidine and uracil or histidine and leucine. The cultures were then diluted to A_{600} = 0.1 with the appropriate synthetic medium containing 2% galactose. At various time intervals, cell growth was monitored by measuring cell number, optical density at 600 nm and colony forming units on agar containing medium lacking uracil, histidine and uracil or histidine and leucine with 2% glucose or 2% galactose as carbon sources.

Preparation of cell extracts

Fractionation of yeast was performed using 40 ml of cells grown in synthetic medium (Sherman, 1991) containing 2% galactose and lacking the appropriate amino acids at various times. Samples (40 ml) of cells were harvested and resuspended in 1 ml cold 1 M sorbitol. Zymolase (20 μg/ml) was added and cells were incubated at 30°C for 45 min. Spheroplasts were washed in 1 M sorbitol and the pellets were resuspended in 0.2 ml lysis buffer [20 mM Tris–HCl pH 7.5, 50 mM KCl, 40 mM NaCl, 5 mM β-mercaptoethanol, 10% Triton X-100, 2 mM phenylmethylsulfonyl fluoride (PMSF)]. After incubation on ice for 10 min, 1/2 vol of glass beads were added and cells were vortexed three times for 20 s at 4°C. Extracts were cleared by two sequential centrifugations (1500 *g* for 5 min and 21 000 *g* for 15 min). Supernatants were flash-frozen and stored at −70°C. Mammalian cell extracts from T98G and Daudi cells were prepared as previously described (Galabru and Hovanessian, 1987).

Polysome analysis

Yeast culture (400 ml) containing each of the expression plasmids was grown overnight at 30°C in synthetic medium containing either 2% glucose or galactose as carbon source. Cells from one half of the culture were harvested (OD_{600} = 0.5), washed and resuspended in 200 ml synthetic medium with 2% galactose. The other half of the culture was diluted to an OD_{600} = 0.25 with synthetic glucose medium. Both cultures were incubated at 30°C for 6 h. Cell extracts were then prepared as described (Baim *et al.*, 1985) and 50 OD_{260} units of each extract was layered on top of a 35 ml linear sucrose gradient (7–47%) containing 50 mM Tris–acetate (pH 7.0), 50 mM NH_4Cl, 12 mM $MgCl_2$, 1 mM dithiothreitol, 0.1% diethylpyrocarbonate and 1 mM PMSF. The gradient was prepared in a stepwise fashion (7 ml each of 17, 27, 37 and 47% sucrose to an initial 7 ml of 7% sucrose) and allowed to equilibrate overnight at 4°C. The gradients were centrifuged at 27 000 r.p.m. in an AH629 rotor for 4 h, collected from the top using an ISCO 640 density gradient fractionator and monitored by an ISCO UA-5 absorbance-fluorescence detector. The positions of the 40S and 60S ribosomal subunits within the polysome gradients have been determined previously (Yoon and Donahue, 1992).

Immunoblotting and autophosphorylation assay

Protein from each sample (120 μg) was electrophoresed on SDS–PAGE and blotted to nitrocellulose membranes. Blots were incubated with a p68 kinase monoclonal antibody (mAb 71/10), washed, incubated with 10^6 c.p.m. per ml of ^{125}I-labeled goat anti-mouse IgG and visualized by autoradiography. For the standard autophosphorylation assay, 150 μg of yeast extract were immunoprecipitated with mAb 71/10 as previously described (Galabru and Hovanessian, 1987) or with influenza hemagglutinin monoclonal antibody 12CA5 (Berkeley Antibody Company) and protein G–Sepharose (Pharmacia). *In vitro* kinase assays were performed without activator as previously described (Galabru and Hovanessian, 1987). 0.5 μg of human eIF-2α was used for substrate phosphorylation assays.

Activation of mammalian extracts using yeast extracts

Mammalian cellular extracts were prepared, immunoprecipitated using mAb–Sepharose and washed as previously described (Galabru and Hovanessian, 1987). Yeast cellular extracts were then added to mAb–Sepharose bound p68 kinase for 2–4 h at 4°C. Samples were washed and *in vitro* phosphorylation assays were performed without activator (Galabru and Hovanessian, 1987).

In vitro translation, dsRNA binding and anti-p68–Sepharose binding assays

Transcription vectors carrying the wild-type (86) and truncated (7, k, see Figure 1) p68 kinase cDNAs were linearized with *Hin*dIII and transcribed *in vitro* with T7 polymerase as described (Meurs *et al.*, 1990). *In vitro* translation for a 50 μl reaction was performed in a rabbit reticulocyte lysate system using 1 μg/ml cRNA in the presence of 75 μCi [^{35}S]methionine. 4 μl were analyzed by SDS–PAGE and 30 μl were used for each of the binding assays. Poly I:poly C cellulose and mAb–Sepharose binding assays were performed as previously described (Meurs *et al.*, 1990).

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